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Design, Synthesis, and Chemical and Biological Properties of Cyclic ADP-4-Thioribose as a Stable Equivalent of Cyclic ADP-Ribose

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Here we describe the successful synthesis of cyclic ADP-4-thioribose (cADP₄T_R, **3**), designed as a stable mimic of cyclic ADP-ribose (cADPR, **1**), a Ca²⁺-mobilizing second messenger, in which the key N1-β-thioribosyladenosine structure was stereoselectively constructed by condensation between the imidazole nucleoside derivative **8** and the 4-thioribosylamine **7** via equilibrium in **7** between the α-anomer (7α) and the β-anomer (7β) during the reaction course. cADP₄T_R is, unlike cADPR, chemically and biologically stable, while it effectively mobilizes intracellular Ca²⁺ like cADPR in various biological systems, such as sea urchin homogenate, NG108-15 neuronal cells, and Jurkat T-lymphocytes. Thus, cADP₄T_R is a stable equivalent of cADPR, which can be useful as a biological tool for investigating cADPR-mediated Ca²⁺-mobilizing pathways.

Keywords: Cyclic ADP-Ribose, cADPR, Second Messenger, Ca²⁺-Release.

INTRODUCTION

Cyclic ADP-ribose (cADPR, **1**, Fig. 1), a metabolite of NAD⁺ (nicotinamide adenine dinucleotide), was originally isolated from sea urchin by Lee and co-workers (Clapper et al., 1987). cADPR mobilizes intracellular Ca²⁺ in various mammalian cells, such as, pancreatic β-cells, smooth muscle, cardiac muscle, T-lymphocytes, and cerebellar neurons, and therefore, cADPR is now recognized as a general mediator of intracellular Ca²⁺ signaling (Galione, 1993; Lee, 1997; Galione et al., 1998; Guse, 1999; Higashida et al., 2001; Lee, 2002; Guse, 2004;

Zhang and Li, 2006; Jin et al., 2007; Venturi et al., 2010; Lee, 2012).

Analogues of cADPR have been extensively designed and synthesized, since they are potentially useful for investigating the mechanism of cADPR-mediated Ca²⁺ release (Walseth and Lee, 1993; Lee et al., 1993; Zhang and Sih, 1995; Ashamu et al., 1995; Wagner et al., 2003, 2005; Moreau et al., 2006, 2012; Galeone et al., 2002; Huang et al., 2002; Gu et al., 2004; Xu et al., 2006; Swarbrick and Potter, 2012; Yu et al., 2012; Shuto et al., 1998; Fukuoka et al., 2000; Shuto et al., 2001; Guse et al., 2002; Shuto et al., 2003; Kudoh et al., 2005; Hashii et al., 2005; Kudoh et al., 2008; Zhang et al., 1999; Shuto and Matsuda, 2004; Guse, 2004; Potter and Walseth, 2004). cADPR analogues are also expected to be lead structures for

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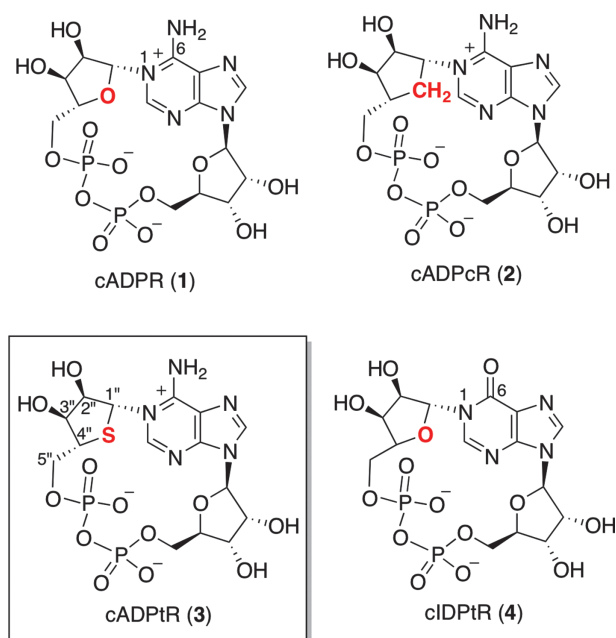


Figure 1. cADPR (1), cADPcR (2), cADPtR (3) and cIDPR (4).

the development of potential drug candidates, due to the important physiological roles of cADPR (Galione, 1993; Lee, 1997; Galione 1998; Guse, 1999; Higashida et al., 2001; Lee, 2002; Guse, 2004; Zhang, 2006; Jin et al., 2007; Venturi et al., 2010; Lee, 2012). cADPR analogs have been synthesized predominantly by enzymatic and chemo-enzymatic methods using ADP-ribosyl cyclase-catalyzed cyclization under mild conditions (Walseth and Lee, 1993; Lee et al. 1993; Zhang and Sih, 1995; Ashamu et al., 1995; Wagner et al., 2003, 2005; Moreau et al., 2006, 2012). The analogs obtained by these methods, however, are limited due to the substrate-specificity of the ADP-ribosyl cyclase (Zhang et al., 1999; Shuto and Matsuda, 2004; Guse, 2004; Potter and Walseth, 2004).

In recent years, methods for the chemical synthesis of cADPR analogs have been studied to develop useful cADPR analogs (Galeone et al., 2002; Huang et al., 2002; Gu et al., 2004; Xu et al., 2006; Swarbrick et al., 2012; Yu et al., 2012; Shuto et al., 1998; Fukuoka et al., 2000; Shuto et al., 2001; Guse et al., 2002; Shuto et al., 2003; Kudoh et al., 2005; Hashii et al., 2005; Kudoh et al., 2008; Zhang et al., 1999; Shuto et al., 2004; Gusu et al., 2004; Potter et al., 2004). Construction of the characteristic pyrophosphate-containing 18-membered ring structure is the key to the synthesis of cADPR and its analogs, and we have developed an efficient method for forming the ring using *S*-phenyl phosphorothioate-type substrates, which can be effectively activated by Ag⁺ or I₂ (Fukuoka et al., 2000; Shuto et al., 2001).

cADPR is readily hydrolyzed at the unstable N1-ribosyl linkage to produce ADP-ribose (ADPR), even in neutral aqueous solution (Lee and Aarhus, 1993). This is due to the fact that cADPR is in a zwitterionic form positively

charged around the N1-C6-N⁶ moiety ($pK_a = 8.3$), making the molecule unstable, where the charged adenine moiety attached to the anomeric carbon of the N1-linked ribose is able to be an efficient leaving group. Under physiological conditions, cADPR is also hydrolyzed at the same N1-ribosyl linkage by cADPR hydrolase to give the inactive ADPR (Lee and Aarhus, 1993).

We previously designed and synthesized cyclic ADP-carbocyclic-ribose (cADPcR, **2**) as a stable mimic of cADPR, in which the oxygen atom in the N1-ribose ring of cADPR is replaced by a methylene group. cADPcR is both chemically and biologically stable, and effectively mobilizes intracellular Ca²⁺ in sea urchin eggs (Shuto et al., 2001). Although cADPcR is more potent than cADPR in neuronal cells (Hashii et al., 2005), it is almost inactive in T cells, whereas natural cADPR effectively mobilizes Ca²⁺ in both neuronal cells and T cells (Guse et al., 2002; Kudoh et al., 2005).

Although intensive studies of the signaling pathway that uses cADPR are still needed because of its biological importance, the biological as well as chemical instability of cADPR limits further studies of its physiological role. Therefore, stable analogs of cADPR exhibiting Ca²⁺-mobilizing activity in various cells including T cells are required. Thus, we newly designed and synthesized a 4-thioribose-containing analog of cADPR, i.e., cyclic ADP-4-thioribose (cADPtR, **3**), in which the N1-ribose of cADPR is replaced by a 4-thioribose. Evaluations in several biological systems revealed that cADPtR is a stable equivalent of cADPR, which mobilizes intracellular Ca²⁺ not only in sea urchin eggs and neuronal cells, but also in T cells. Here we report the detailed results of these studies on cADPtR.

RESULTS AND DISCUSSION

Design of cADPtR

cADPR is in an equilibrium between the N⁶-protonated amino-form and the N⁶-deprotonated imino-form, as shown in Figure 2 (Lee et al., 1989; Kim et al., 1993; Lee et al., 1994; Gu and Sih, 1994; Wada et al., 2005; Wada and Pope, 2001; Graham et al., 2004). One difference between cADPR and cADPcR is their pK_a values for protonation at the N⁶-position. The pK_a of cADPcR (8.9) (Guse et al., 2002) is somewhat higher than that of cADPR (8.3) (Kim et al., 1994), which might affect its interaction with target proteins. Under physiological conditions, cADPR is in a mixture of the protonated amino form and the deprotonated imino form, where most of cADPcR should be present in the amino-form due to its relatively higher pK_a . If cADPR binds to the target protein in T cells in the imino-form, activity of cADPcR would be decreased. In recent years, cADPR analogs with a hypoxanthine base instead of an adenine base, such as cyclic IDP-ribose (cIDPR, **4**, Fig. 1), were synthesized,

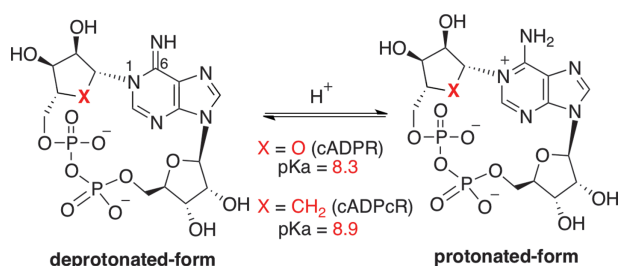


Figure 2. Equilibrium between the N⁶-protonated amino-form and the N⁶-deprotonated imino-form in cADPR and cADPCr.

and these hypoxanthine-type analogs also mobilized Ca²⁺ in T cells, while they were almost inactive in other biological systems (Moreau et al., 2006; Huang et al., 2002; Gu et al., 2004; Xu et al., 2006). These findings support the idea of an active imino-form of cADPR in T cells, because the C6=O⁶ moiety might work as a bioisostere of the plane C6=N⁶ moiety of the imino-form of cADPR, due to their analogous unsaturated π -electronic structural features.

Another difference between cADPR and cADPCr might be their three-dimensional structures. In nucleosides, conformation around the glycoside linkage is one of the determinants of their biological activities, and they generally prefer a conformation that avoids steric repulsion between the nucleobase and sugar moieties (Saenger, 1983). Therefore, in cADPR and its analogs, the most stable conformation can be the one with minimal steric repulsion between the adenine moiety and both of the N1- and N9-ribose moieties. It should be noted that, in cADPCr, the hydrogens on the tetrahedral sp³-C6'', particularly the H6''- β , which is absent in cADPR, are rather sterically repulsive to the adenine H2 (Fig. 3). Accordingly, the stable conformation of cADPCr would differ from that of cADPR due to the steric effects (Kudoh et al., 2008).

Taking these considerations into account, we newly designed cADPr, a 4-thioribose analog of cADPR. 4''-Thionucleosides are recognized as useful bioisosteres of the natural nucleosides, which are extensively used in studies of medicinal chemistry and chemical biology (Reist et al., 1964; Bobek and Whistler, 1970; Dyson et al., 1991; Bellon et al., 1993; Yoshimura et al., 1997; Naka et al., 2000; Takahashi et al., 2009). The 4''-thionucleosides does not only effectively mimics the ribose

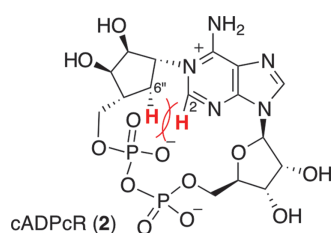


Figure 3. Possible steric repulsion between the H2 and the H6''- β .

of nucleosides, but also the N-4-thioribosyl linkage is more stable than the corresponding N-ribose linkage against both chemical and enzymatic hydrolysis (Elzagheid et al., 1999; Toyohara et al., 2003). In addition, the pK_a value of cADPr is expected to be similar to that of cADPR due to the electron-withdrawing property of sulfur atom. This type of pK_a adjustment of biologically active compounds by replacing a methylene with a sulfur has been reported previously (Ganellin and Owen, 1977). Furthermore, the conformation of cADPr, particularly spatial positioning of the N1-thioribose and adenine moieties, would be similar to that of cADPR, because both sulfur and oxygen have a similar sp³ configuration bearing two non-bonding electron pairs. Thus, we expected that cADPr might be a stable cADPR equivalent that is active in various cells including T cells.

Synthetic Plan

The retrosynthetic scheme for the target cADPr (3) is shown in Figure 4. For the synthesis of cADPr, construction of the 18-membered pyrophosphate structure is an important step. The structure was likely to be constructed using the intramolecular condensation reaction with an *S*-phenyl phosphorothioate-type substrate that we developed (Fukuoka et al., 2000; Shuto et al., 2001), which has been effectively used in the synthesis of a variety of cADPR analogs (Galeone et al., 2002; Huang et al., 2002; Gu et al., 2004; Xu et al., 2006; Swarbrick et al., 2012; Yu et al., 2012). Thus, treatment of the *S*-phenyl phosphorothioate-type substrates 5 having the N1-4-thioribosyladenosine structure with AgNO₃/MS3A as a promoter (Fukuoka et al., 2000; Shuto et al., 2001) would form the desired cyclization product, and subsequent deprotection would furnish the target cADPr. The *S*-phenyl phosphorothioate-type substrates 5 would be obtained from the N1- β -thioribosyl adenosine derivative 6 β , which we planned to construct by condensation between the 4-thioribosylamine 7 and a known imidazole nucleoside derivative 8 (Hutchinson et al., 1997) readily prepared from inosine. The 4-thioribosylamine 7 was expected to be prepared from the 1-deoxy-4-thioribose derivative 9, obtained by a known method (Jeong et al., 2006).

In this synthetic plan, we first had to achieve stereoselective construction of the N1- β -thioribosyladenosine structure. Although no 4-thioribose derivatives having an anomeric amino function such as 7 have been reported to date, 4-thioribosylamine 7 is likely to be present as an anomeric mixture, due to the electron-donating property of the hemiaminal ether nitrogen attaching at the anomeric position. We speculated that if the 4-thioribosylamine derivative 7 is indeed in equilibrium between the α -anomer (7 α) and the β -anomer (7 β), stereoselective construction of the N1- β -thioribosyladenosine structure might be

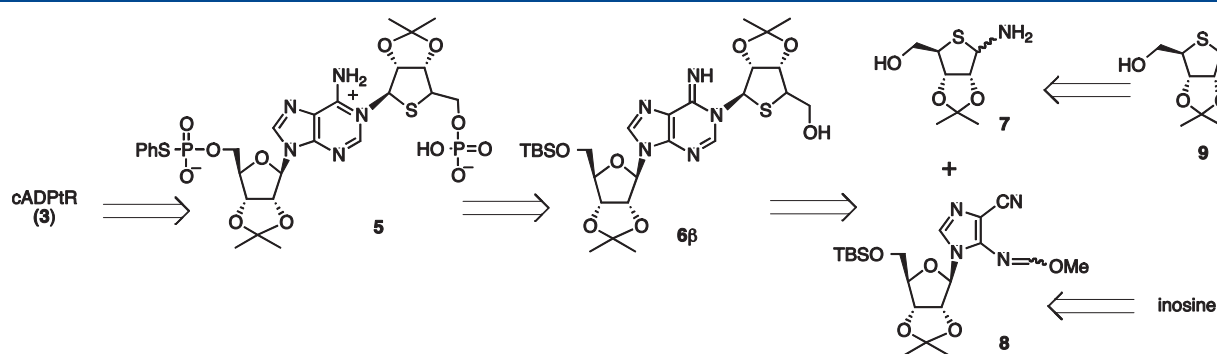


Figure 4. Retrosynthetic analysis of the target cADP4r (3).

achieved. In the 4-thioribosylamine **7**, the α -face would be rather sterically more hindered than the β -face due to its rigid 5,5-*cis* ring system. Thus, as shown in Figure 5, in the condensation reaction, the β -anomer **7β** might preferentially react with the imidazole nucleoside **8**, while the α -anomer **7α** might not react due to the steric hindrance by the adjacent isopropylidene moiety. Thus, we expected that, in the reaction course, the relatively less reactive α -anomer would not undergo the condensation, but rather would be effectively converted into the more reactive β -anomer via the equilibrium to result in accumulation of the desired β -condensation product **6β**.

Synthesis

The 4-thioribosylamine **7** was prepared from the known 1-deoxy-4-thioribose derivative **9** (Jeong et al., 2006), as shown in Scheme 1. While several procedures for synthesizing 4-thioribose derivatives have been reported (Reist et al., 1964; Bobek and Whistler, 1970; Dyson et al., 1991; Bellon et al., 1993; Yoshimura et al., 1997; Naka et al., 2000; Takahashi et al., 2009), we selected the procedure recently developed by Jeong and co-workers (Jeong et al., 2006), which is effective for large scale preparation of the 4-thio-1-deoxyribose derivative **9** having the desired 2,3-*O*-isopropylidene protecting group. Oxidation of **9** with

m-CPBA and subsequent heating of the resulting sulfoxide product in Ac_2O to initiate the Pummerer rearrangement afforded the 1-acetoxy product **11** as an anomeric mixture ($\alpha/\beta = 1:5$). When the anomeric mixture **11** was treated with TMSN_3 and SnCl_4 in CH_2Cl_2 , the β -azide **12** was obtained stereoselectively in high yield, probably due to the steric demand of the reaction intermediate. Reduction of the azido group of **12** by catalytic hydrogenation, followed by removal of the *O*-acetyl group by heating it in MeOH gave the 4-thioribosylamine **7**, which appeared to be an anomeric mixture ($\alpha/\beta = 1:2$) as we expected.

We next investigated the key condensation between the 4-thioribosylamine **7** ($\alpha/\beta = 1:2$) and the imidazole nucleoside **8** under various conditions. Under basic reaction conditions, i.e., $\text{K}_2\text{CO}_3/\text{MeOH}$ which was previously used to construct the N1-carbocyclic-ribosyladenine structures (Shuto et al., 2001), the desired β -condensation product **6β** was actually obtained stereoselectively in 50% yield. Under the conditions, however, the N^6 -(4-thioribosyl) product **13** via a Dimroth rearrangement was also obtained in 28% yield. As a result, we found that when **7** was treated with **8** (2.1 eq) in MeOH at room temperature without any bases, the β -product **6β** was obtained in 61% yield concomitant with 5% of the α -product **6α**, where the 4-thioribosylamine **7** was recovered in 17% yield (Scheme 2). Thus, the desired β -product **6β** was successfully obtained in 73% conversion yield from the

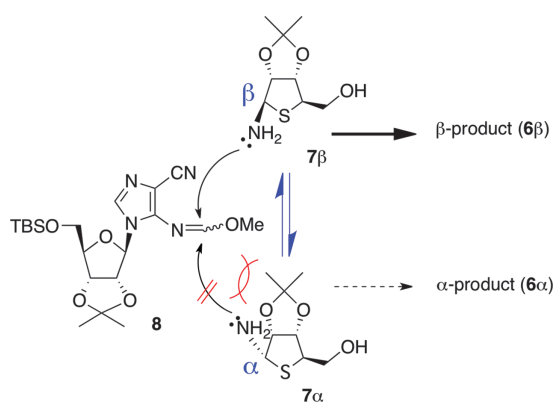
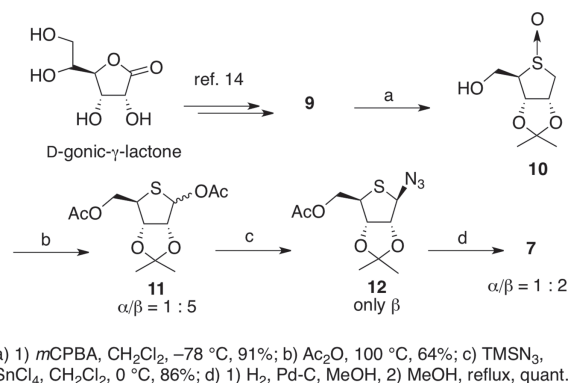
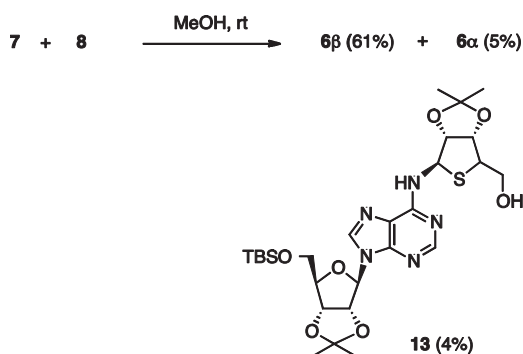


Figure 5. Hypothesis for the stereoselective formation of the desired β -product **7β** via the α/β equilibrium.



Scheme 1. Synthesis of 4-thioribosylamine **7**.



Scheme 2. Stereoselective synthesis of N1- β -thioribosyladenosine derivative **6 β** .

4-thioribosylamine ($\alpha/\beta = 1:2$) via the α/β -equilibrium, as we hypothesized. In the reaction conditions, the N 6 -thioribosyl product **13** (4%) was also obtained.

The N1-substituted structures of **6a** and **6b** was confirmed based on their HMBC spectra, in which correlation between the H2 of the adenine and the C1'' of the 4-thioribose moiety was observed. The α - and β -stereochemistries of **6a** and **6b** were confirmed by NOE data (see Fig. 11).

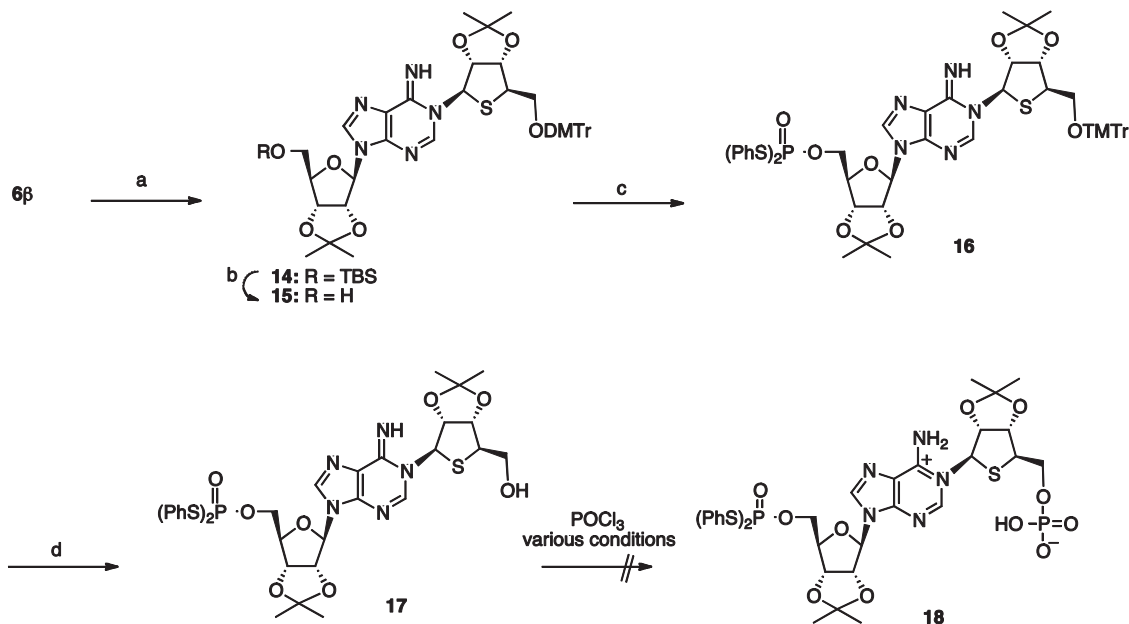
Conversion of the N1- β -thioribosyl product **6b** into the *S*-phenyl phosphorothioate-type substrate **5** was next investigated (Scheme 3). The 5''-hydroxy group of **6b** was protected with a dimethoxytrityl (DMTr) group, and then the 5''-O-TBS group of the product **14** was removed with TBAF to give **15**. Treatment of **15** with an *S,S'*-diphenylphosphorodithioate

(PSS)/2,4,6-triisopropylbenzenesulfonyl chloride (TPSCI)/pyridine system (Sekine et al., 1985; Sekine and Hata, 1993), followed by removal of the 5''-O-DMTr group of the product **16** with aqueous AcOH gave the 5''-bis-*S*-(phenyl)phosphorothioate **17**.

We next examined phosphorylation of the 5''-hydroxyl of **17**. However, treatment of **17** under the usual conditions of Yoshikawa's phosphorylation procedure with $\text{POCl}_3/(\text{EtO})_3\text{PO}$ (Yoshikawa et al., 1969), which was effective in the previous synthesis of cADPcR and its analogs, gave none of the desired phosphorylation product **18**.

Difficulties in the phosphorylation of the 4-thioribose moiety could be assumed to occur, at least partially, because phosphorylation of the 5''-primary hydroxyl of 4'-thioribonucleoside derivatives is rather difficult compared to the cases with usual ribonucleosides (Hancox and Walker, 1996; Alexandova et al., 1996). Walker and co-workers reported that phosphorylation of 4'-thiothymidine by Yoshikawa's procedure did not give the desired corresponding 5''-O-phosphate product. They suggested that elimination of the dichlorophosphate moiety would occur in an intermediate **A** due to effective neighboring group participation by the nucleophilic ring sulfur to result in reproducing the starting material 4'-thiothymidine, via a thionium intermediate **B** and its subsequent hydrolysis, as shown in Scheme 4(a) (Hancox and Walker, 1996).

We thought that a zwitterionic phosphorylating reagent **19** (Scheme 4(b)), reported by Asseline and Thuong (Asseline and Thuong et al., 1988), might be effective for the phosphorylation of **17**, because in the phosphorylation



Reagents and conditions: a) DMTrCl, pyridine, rt, 81%; b) TBAF, THF, AcOH, rt, quant; c) PSS, TPSCI, pyridine, -15°C , 72%; d) aq. AcOH, 90%.

Scheme 3. Investigation for synthesizing 5'-bis-*S*-(phenyl)phosphorothioate **17**.

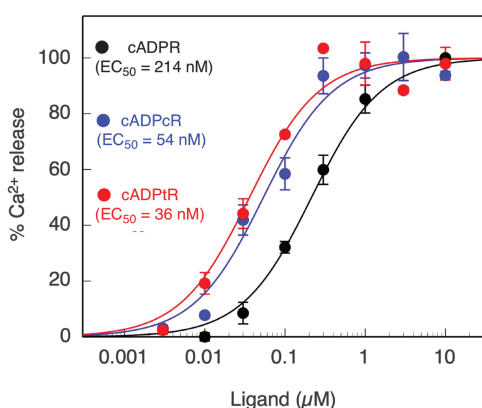


Figure 6. Concentration-dependent Ca^{2+} -mobilizing activity of cADPR, cADPcR, and cADPtR in sea urchin egg homogenate. The Ca^{2+} -mobilizing activity of each compound is expressed as the percent change in ratio of fura-2 fluorescence (F340/F380) relative to that of 10 μM cADPR. Data are mean \pm SEM of 3 to 6 experiments.

not cause an increase in $[\text{Ca}^{2+}]_i$ in the conditions in which the plasma membrane was permeabilized with 250 nM of digitonin for nearly 5 min (Figs. 7(b) and (c)). We also confirmed that application of 100 mM cADPR (**1**), used as a positive control, caused a gradual and sustained Ca^{2+} release in the digitonin-permeabilized conditions, which allowed nucleotides to enter into the cytoplasm (Fig. 8(c)). When cADPR was applied extracellularly to intact NG108-15 cells, no increase in $[\text{Ca}^{2+}]_i$ was detected (data not shown). Figure 7(a) shows a representative field of cells with $[\text{Ca}^{2+}]_i$ changes induced by the application of cADPtR with the plasma cell membrane was permeabilized with 250 nM digitonin for 5 min. Figure 8(c) shows the mean time-course of $[\text{Ca}^{2+}]_i$ changes induced by cADPtR. Application of 100 μM cADPtR induced persistent increases in $[\text{Ca}^{2+}]_i$; the mean $[\text{Ca}^{2+}]_i$ level measured 4 min after application of cADPtR was $116 \pm 2.3\%$ of the resting (pre-permeabilization) level (mean \pm SEM; $n = 6$). The amplitude evoked by cADPtR was equivalent to or significantly greater than that induced by cADPR: the mean $[\text{Ca}^{2+}]_i$ level measured 4 min after application of cADPR was $107 \pm 2.8\%$ of the resting level ($n = 10$). A similar Ca^{2+} -mobilizing pattern was observed in cADPcR-applied cells: the mean $[\text{Ca}^{2+}]_i$ level measured 4 min after application of cADPcR was $115 \pm 3.1\%$ of the resting level ($n = 10$).

These results in NG108-15 neuronal cells indicate that cADPtR has potent Ca^{2+} -mobilizing activity, similar to cADPcR. Both cADPtR and cADPcR have increased efficacy as compared with cADPR, which may be due to their increased metabolic stability.

Ca^{2+} -Mobilizing Activity in *T* Cells

The Ca^{2+} -mobilizing effect of cADPtR (**3**) was evaluated using saponin-permeabilized Jurkat *T* cells as reported

previously (Guse et al., 1993; Guse et al., 2007; Schwarzmann et al., 2002). Both cADPtR and cADPR (**1**) evoked rapid Ca^{2+} release upon addition to the permeabilized cell suspension indicating similar mechanisms of Ca^{2+} release (Fig. 8(a)). Though cADPR was somewhat more potent at 100 mM, cADPR and cADPtR had very similar concentration-response curves (Fig. 8(b)) indicating that cADPtR binds to the cADPR receptor protein with similar affinity. Our previous work revealed that replacing the northern ribose of cADPR with the carbocyclic moiety (cADPcR) shifted its Ca^{2+} mobilizing activity to much higher concentrations (Guse et al., 2002). In contrast, the new analog cADPtR was almost as active as cADPR. A difference of cADPcR and analog cADPtR are their pK_a values, amounting to 8.9 and 8.0, respectively. Taking the pK_a value of cADPR and the indistinguishable biological activity between cADPR (**4**) and cADPR (Moreau et al., 2006; Huang et al., 2002; Gu et al., 2004; Xu et al., 2006) in *T* cells into account, the relatively high biological activity of cADPtR might be in favour of the imino form of cADPR being the active form of the natural second messenger in *T* cells.

Conformational Analysis

The three-dimensional structures of biologically active compounds in aqueous solution is very important from the viewpoint of the bioactive conformation. Our study shows that cADPR (**1**), cADPcR (**2**), and cADPtR (**3**) have different activities in sea urchin eggs, neuronal cells and *T* cells, respectively. To investigate the biological difference based on their conformations, structures of them were constructed by molecular dynamics calculations with a simulated annealing method based on the NOE constraints of the intramolecular proton pairs measured in D_2O (for details, see Fig. 13). The structures obtained by the calculations based on their observed NOE in the NOESY spectra are shown in Figure 9.

To analyze the structural differences in more detail, these structures were superimposed as shown in Figure 10(a), which shows that the cADPtR structure (red) resembles that of natural cADPR (blue). The cADPcR structure (green), however, is not similar to those of the other two compounds, in which the relative special arrangement of the N1-thioribose ring and the adenine ring is clearly different from those of the other two compounds, as we expected. Thus, the distances between the 4''S between the adenine H2 of cADPtR (3.6 Å) is significantly longer than the corresponding distances of cADPR (2.3 Å) and cADPcR (2.5 Å), probably due to the steric repulsion between the H6''β with the adenine H2 in cADPcR.

To confirm the validity of the obtained structures, we used the cADPR structure solved by X-ray crystallographic analysis (2j, Lee et al., 1994). Thus, the X-ray

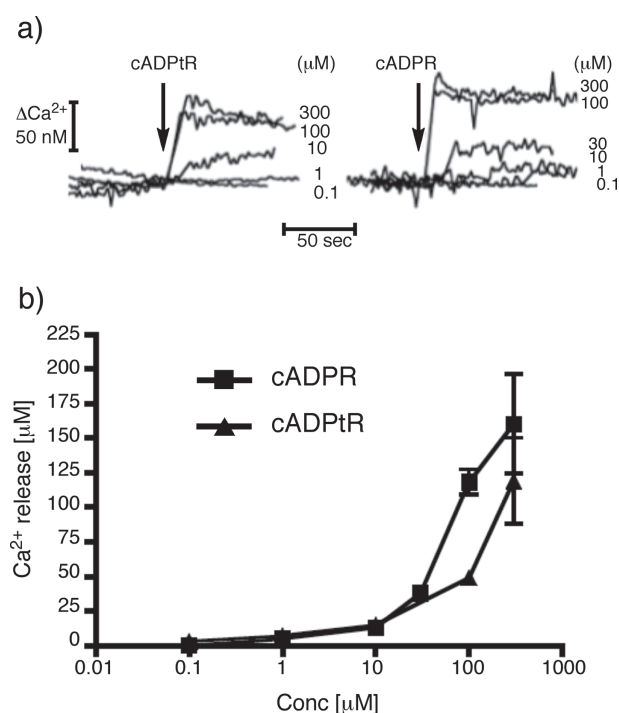


Figure 8. Effect of cADPR (1) and cADPtR (3) on Ca^{2+} signaling in permeabilized Jurkat T cells. After addition of fura-2/free acid (1.5 mM) and charging of the intracellular Ca^{2+} -pools by the addition of ATP (1 mM) and an ATP-regenerating system consisting of creatine phosphate (10 mM) and creatine kinase (20 units/ml), the indicated concentrations of cADPR or cADP-4'-thio-ribose were added as indicated. (a) Representative tracings. (b) Data presented as mean \pm SEM ($n = 2$ to 8).

On the other hand, the sulfur of thio-sugars, including thioribose, would not so effectively stabilize the corresponding thio-carbenium intermediates, because the hyperconjugation ability of sulfur is remarkably weaker than that of oxygen (Salzner and Salzner, 1993), which can be reason why cADPtR is more stable than cADPR.

On the other hand, the electron-withdrawing effect of sulfur due to its electronically negative property effectively lowered the pK_a of cADPtR, and accordingly, its pK_a is similar to that of cADPR. Under physiological pH, the population of the deprotonated imino form and the protonated amino form in cADPtR would be analogous that

in cADPR. Therefore, the pK_a value of cADPtR may be favorable to exhibit its biological effects, similarly to natural messenger cADPR.

A difference of cADPcR and analog cADPtR are their pK_a values, amounting to 8.9 and 8.0, respectively. Taking the pK_a value of cADPR and the indistinguishable biological activity between cIDPR (4) and cADPR (Moreau et al., 2006; Huang et al., 2002; Gu et al., 2004; Xu et al., 2006) in T cells into account, the relatively high biological activity of cADPtR might be in favour of the imino form of cADPR being the active form of the natural second messenger in T cells.

Our conformational analysis revealed that cADPtR has its three-dimensional structure analogous to cADPR due to the similar sp^3 configuration of oxygen and sulfur bearing two non-bonding electron pairs in the N1-ribose or -thioribose ring, in which steric repulsion between the three rings (adenine, N9-ribose, and N1-ribose) seems to be minimal. Therefore, the structural and electrostatic features of cADPtR analogous to cADPR make it as biologically active as cADPR in various systems including T-cells, although the target proteins of cADPR in these systems is considered to be different (Kudoh et al., 2005).

Conclusion

We have successfully synthesized cADPtR (3) and demonstrated that it is stable and functions as cADPR (1) in various biological systems, i.e., sea urchin egg homogenate, neuronal cells and T cells. Because of the characteristic stability and high potency of cADPtR, it, as a stable equivalent of cADPR, can be useful as an effective tool for identifying target proteins and for investigating cADPR-mediated signaling pathways (Tsuzuki et al., 2013).

EXPERIMENTAL DETAILS

Chemical shifts are reported in ppm downfield from Me_4Si (^1H), MeCN (^{13}C) or H_3PO_4 (^{31}P). All of the ^1H NMR assignments described were in agreement with COSY spectra. Thin-layer chromatography was done on Merck coated plate 60F $_{254}$. Silica gel chromatography was done

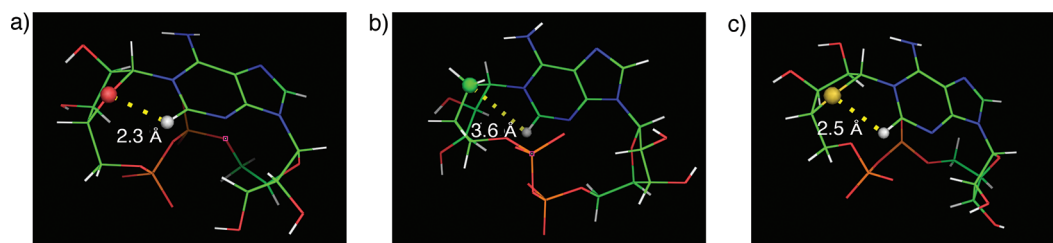


Figure 9. Structures of cADPR (a), cADPcR (b), cADPtR (c) by molecular dynamics calculations with simulated annealing method using the NOE data in D_2O . Adenine H2 (white), O4'' in cADPR (red), C6'' in cADPcR (green) and S4'' in cADPtR (yellow) are shown in sphere.

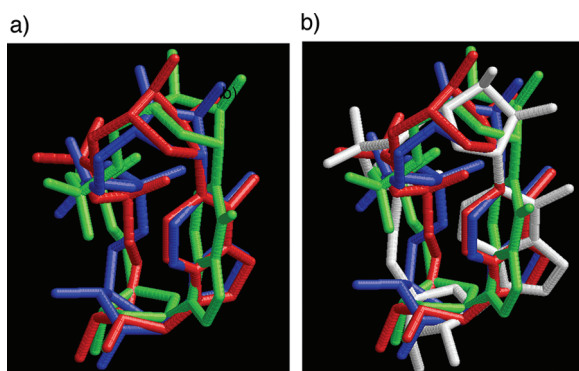


Figure 10. Superimposed displays of the structures of cADPR and its analogs. Only heavy atoms are shown. **(a)** Three calculated structures are superimposed: cADPR (blue), cADPrR (green), and cADPrR (red). **(b)** Four structures were superimposed: The crystal structure of cADPR (white), the calculated structures of cADPR (blue), cADPrR (green), and cADPrR (red).

on Merck silica gel 5715. Reactions were carried out under an argon atmosphere.

1,4-Dideoxy-1,4-Episulfinyl-2,3-O-Isopropylidene-D-Ribitol (10)

To a solution of **22** (1.90 g, 10.0 mmol) in CH_2Cl_2 (30 mL) was added slowly a solution of *m*CPBA (3.18 g, 12.0 mmol) in CH_2Cl_2 (60 mL) at -78°C , and the mixture was stirred at the same temperature for 20 min. To the mixture was added aqueous $\text{Na}_2\text{S}_2\text{O}_3$, and the resulting mixture was partitioned between EtOAc and H_2O , and the organic layer was washed with aqueous saturated NaHCO_3 and brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (SiO_2 , hexane/AcOEt = 3/1 then 1/1) to give **10** (1.88 g, 91%, white amorphous solid): $^1\text{H-NMR}$ of one isomer (500 MHz, CDCl_3) δ 1.33 (3 H, *s*, isopropylidene- CH_3), 1.47 (3 H, *s*, isopropylidene- CH_3), 3.29 (1 H, dd, J = 14.3, 6.3 Hz, H-1), 3.42 (1 H, ddd, J = 5.7, 5.7, 2.9 Hz, H-4), 3.45 (1 H, dd, J = 14.3, 5.7 Hz, H-1) 4.10 (1 H, dd, J = 12.6, 5.7 Hz, H-5), 4.34 (1 H, dd, J = 12.6, 2.9 Hz, H-5), 4.80 (1 H, br, OH), 5.10 (1 H, dd, J = 5.7, 3.4 Hz,

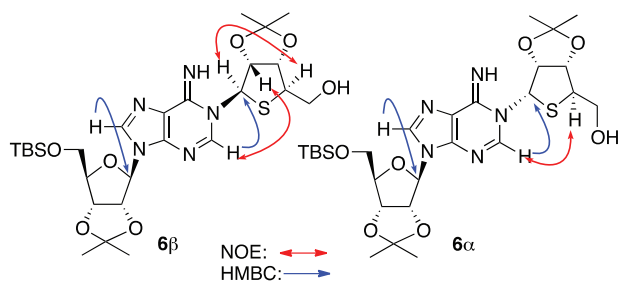


Figure 11. NOE and HMBC data of **6b** and **6a**.

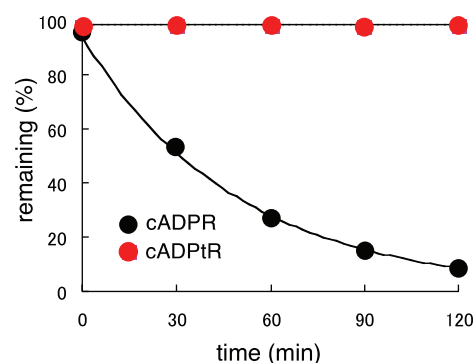


Figure 12. Stability of cADPrR and cADPr in rat brain microsome extract.

H-3), 5.20 (1 H, ddd, J = 6.3, 5.7, 3.4 Hz, H-2); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ 24.61, 27.12, 57.06, 57.06, 58.11, 65.63, 79.76, 82.22, 112.11; HR-MS (EI) calcd for $\text{C}_8\text{H}_{14}\text{O}_4\text{S}_2$ 206.06128 (M^+), found 206.06169.

1,5-O-Diacetyl-2,3-O-Isopropylidene-4-Thio-D-Ribose (11)

A solution of **10** (135 mg, 0.44 mmol) in Ac_2O (3 mL) was heated at 100°C for 28 h, and then evaporated. The residue was partitioned between EtOAc and H_2O , and the organic layer was washed with aqueous saturated NaHCO_3 and brine. The aqueous layers were combined and extracted with CHCl_3 . The organic layers were combined, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (SiO_2 , hexane/AcOEt = 6/1, 3/1 then 1/1) to give **11** (93 mg, 64%, J = 11.5, 5.7 Hz, H-5), 4.89–4.91 (2 H, m, H-2, H-3), 6.04 (1 H, *s*, H-1), for α -anomer, δ 1.36 (3 H, *s*, isopropylidene- CH_3), 1.54 (3 H, *s*, isopropylidene- CH_3), 2.10 (3 H, *s*, Ac), 2.15 (3 H, *s*, Ac), 3.90 (1 H, m, H-4), 4.19 (1 H, dd, J = 11.5, 6.3 Hz, H-5), 4.35 (J = 11.5, 5.7 Hz, H-5), 4.89–4.91 (2 H, m, H-2, H-3), 6.04 (1 H, *s*, H-1), for α -anomer, δ 1.36 (3 H, *s*, isopropylidene- CH_3), 1.54 (3 H, *s*, isopropylidene- CH_3), 2.10 (3 H, *s*, Ac), 2.15 (3 H, *s*, Ac), 3.90 (1 H, m, H-4), 4.19 (1 H, dd, J = 11.5, 6.3 Hz, H-5), 4.35 (1 H, dd, J = 11.5, 6.3 Hz, H-5), 4.67 (1 H, dd, J = 6.3, 4.0 Hz, H-3), 4.88 (1 H, m, H-2), 6.09 (1 H, d, J = 5.2 Hz, H-1) $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 20.85, 21.21, 24.61, 26.38, 53.85, 65.71, 85.13, 87.17, 88.54, 111.29, 169.16, 170.49; HR-MS (FAB, positive) calcd for $\text{C}_{12}\text{H}_{18}\text{NaO}_6\text{S}$ 313.0722 [$(\text{M}+\text{Na})^+$], found 313.0717.

5-O-Acetyl-2,3-O-Isopropylidene-4-Thio- β -D-Ribofuranosylazide (12)

To a solution of **11** (2.26 g, 7.78 mmol) and TMSN_3 (3.09 mL, 23.3 mmol) in CH_2Cl_2 (20 mL) was added a solution of SnCl_4 (223 mL, 1.91 mmol) in CH_2Cl_2 (10 mL) at 0°C , and the mixture was stirred at the

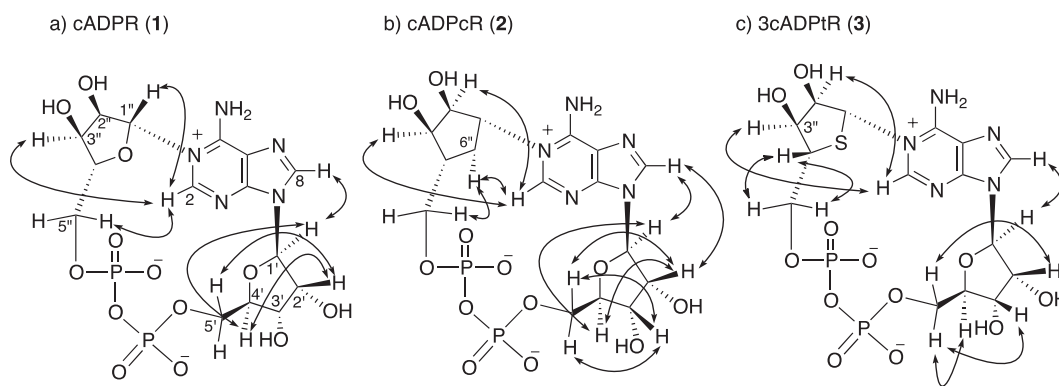


Figure 13. Important correlations in NOESY spectra of (a) cADPR (1), (b) cADPCr (2), (c) cADPTr (3), used for the calculations.

same temperature for 5 min. To the mixture was added aqueous saturated NaHCO_3 , and the resulting white precipitate was filtered off with Celite. The filtrate was washed with aqueous saturated NaHCO_3 and brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (SiO_2 , hexane/ AcOEt = 20/1) to give **12** (1.84 g, 86%, yellow oil): $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 1.31 (3 H, s, isopropylidene- CH_3), 1.50 (3 H, s, isopropylidene- CH_3), 2.11 (3 H, s, Ac), 3.63 (1 H, dd, J = 10.0, 5.4 Hz, H-4), 4.12 (1 H, dd, J = 11.3, 10.0 Hz, H-5), 4.27 (1 H, dd, J = 11.3, 5.4 Hz, H-5), 4.64 (1 H, d, J = 5.4 Hz, H-3), 4.86 (1 H, d, J = 5.4 Hz, H-2), 5.18 (1 H, s, H-1); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 20.73, 24.51, 26.26, 54.29, 65.21, 76.13, 85.59, 89.06, 111.23, 170.40; HR-MS (FAB, positive) calcd for $\text{C}_{10}\text{H}_{15}\text{N}_3\text{NaO}_4\text{S}$ 296.0681 $[(\text{M}+\text{H})^+]$, found 296.0695.

2,3-O-Isopropylidene-4-Thio-D-Ribofuranosylamine (7)

A mixture of **12** (1.25 g, 4.57 mmol) and Pd/C (10%, 630 mg) in MeOH (45 mL) was stirred under atmospheric pressure of H_2 at room temperature for 1 h, and then the catalysts were filtered off with Celite. The filtrate was evaporated, and the residue was purified by column chromatography (NH-silica gel, hexane/ AcOEt = 6/1, 2/1 then 1/3) to give **7** (1.09 g, quant., α/β = 1/2, brown oil): $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ 1.30 (2 H, s), 1.36 (1 H, s), 1.52, (2 H, s), 1.57 (1 H, s), 1.83 (2 H, brs), 3.58 (2/3 H, dd, J = 7.7, 5.4 Hz), 3.73 (1/3 H, ddd, J = 6.3, 5.9 Hz), 4.32 (2/3 H, dd, J = 11.7, 7.7 Hz), 4.43 (2/31 H, dd, J = 11.7, 5.4 Hz), 4.49 (1/3 H, dd, J = 11.3, 5.9 Hz), 4.60–4.66 (2/3 H, m), 4.71–4.74 (1 H, m), 4.79 (2/3 H, d, J = 5.2 Hz, b-H-1), 4.92 (1/3 H, dd, J = 4.5 Hz), 5.04 (2/3 H, d, J = 4.5 Hz); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ -5.64, -5.51, 18.23, 24.36, 25.27, 25.80, 26.50, 27.22, 57.20, 63.68, 64.31, 64.63, 81.06, 85.99, 86.45, 87.57, 91.20, 91.65, 95.13, 110.33, 113.74, 117.26, 132.20, 148.52, 151.04; HR-MS (EI) calcd for $\text{C}_8\text{H}_{15}\text{NO}_3\text{S}$ 205.0773 (M^+), found 205.0773.

N1-(2,3-O-isopropylidene-4-thio- β -D-ribofuranosyl)-5''-O-(*tert*-butyldimethylsilyl)-2',3'-O-Isopropylideneadenosine (6 β)

A solution of **7** (381 mg, 1.87 mmol) and **8** (1.79 g, 4.00 mmol) in MeOH (10 mL) was stirred at room temperature for 10 h, and then evaporated. The residue was purified by flash column chromatography (silica gel, hexane/ AcOEt = 1/2, AcOEt , then AcOEt/MeOH = 9/1) to give **6 β** (700 mg, 61%, white amorphous solid), **6 α** (59 mg, 5%, white amorphous solid), **14** (30 mg, 4%, yellow amorphous solid). **6 β** : $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ 0.04 (3 H, s, Si- CH_3), 0.05 (3 H, s, Si- CH_3), 0.86 (9H, s, *tert*-butyl), 1.35 (3 H, s, isopropylidene- CH_3), 1.39 (3 H, s, isopropylidene- CH_3), 1.61 (3 H, s, isopropylidene- CH_3), 1.63 (3 H, s, isopropylidene- CH_3), 3.77 (1 H, dd, J = 11.4, 4.0, 4.0 Hz, H-5'), 3.83–3.88 (2 H, m, H-5', H-5''), 3.90 (1 H, m, H-4'), 4.01 (1 H, dd, J = 11.4, 3.7 Hz, H-5''), 4.39 (1 H, m, H-4'), 4.88 (1 H, dd, J = 6.3, 3.4 Hz, H-3'), 4.99 (1 H, dd, J = 5.2, 1.7 Hz, H-3''), 5.06 (1 H, dd, J = 6.3, 2.3 Hz, H-2'), 5.51 (1 H, dd, J = 5.2, 3.4 Hz, H-2''), 6.00 (1 H, d, J = 3.4 Hz, H-1''), 6.02 (1 H, d, J = 2.3 Hz, H-1'), 7.85 (1 H, s, H-8), 7.88 (1 H, s, H-2); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ -5.58, -5.47, 18.25, 25.25, 25.80, 27.13, 27.92, 55.53, 63.37, 64.75, 74.54, 81.19, 85.23, 85.75, 86.22, 86.95, 91.15, 111.66, 114.09, 123.75, 137.20, 140.30, 146.81, 153.31; UV (MeOH) λ_{max} = 260 nm; LR-MS (FAB, positive) m/z 610 $[(\text{M}+\text{H})^+]$; Anal. Calcd for $\text{C}_{27}\text{H}_{43}\text{N}_5\text{O}_7\text{SSi}$: C, 53.18; H, 7.11, N, 11.48. Found C, 52.88; H, 6.95; N, 11.35; NOE irradiated H-2'/observed H-2'' (3.8%), irradiated H-1''/observed H-4'' (2.3%), irradiated H-2''/observed H-2 (4.7%), irradiated H-4''/observed H-1'' (3.2%). **6 α** : $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ 0.05 (3 H, s, Si- CH_3), 0.05 (3 H, s, Si- CH_3), 0.87 (9H, s, *tert*-butyl), 1.35 (3 H, s, isopropylidene- CH_3), 1.39 (3 H, s, isopropylidene- CH_3), 1.61 (3 H, s, isopropylidene- CH_3), 1.63 (3 H, s, isopropylidene- CH_3), 3.56 (1 H, d, J = 4.1, 4.1 Hz, H-4''), 3.76 (1 H, dd, J = 11.3, 3.6 Hz, H-5'), 3.84–3.88 (2 H, m, H-5', H-5''), 3.93 (1 H, dd, J = 10.9, 4.1 Hz, H-5''), 4.39 (1 H, ddd, J = 6.3, 3.6, 3.6 Hz, H-4'), 4.90 (1 H, dd, J = 6.3, 2.7 Hz, H-3'),

4.95 (1 H, d, $J = 5.9$ Hz, H-3''), 5.01 (1 H, dd, $J = 5.9$, 4.5 Hz, H-2''), 5.09 (1 H, dd, $J = 6.3$, 2.7 Hz, H-2'), 6.05 (1 H, d, $J = 2.7$ Hz, H-1'), 7.02 (1 H, d, $J = 4.5$ Hz, H-1''), 7.83 (1 H, s, H-8), 8.42 (1 H, s, H-2); ^{13}C -NMR (125 MHz, CDCl_3) δ -5.54, -5.44, 18.29, 24.22, 25.33, 25.83, 25.97, 27.16, 54.36, 61.15, 63.34, 65.17, 80.59, 81.19, 85.30, 85.87, 86.99, 91.16, 111.38, 114.09, 122.77, 136.83, 140.88, 147.76, 155.00; UV (MeOH) $\lambda_{\text{max}} = 259$ nm; HR-MS (FAB, positive) calcd for $\text{C}_{28}\text{H}_{43}\text{N}_5\text{O}_7\text{SSi}$ 610.2731 (MH^+), found 610.2727. NOE irradiated H-2'/observed H-4'' (1.0%), irradiated H-1''/observed H-2'' (10.7%), irradiated H-2''/observed H-1'' (8.4%), irradiated H-4''/observed H-2 (1.7%). **13**: ^1H -NMR (500 MHz, CDCl_3) δ 0.00 (3 H, s, Si- CH_3), 0.01 (3 H, s, Si- CH_3), 0.83 (9H, s, *tert*-butyl), 1.32 (3 H, s, isopropylidene- CH_3), 1.40 (3 H, s, isopropylidene- CH_3), 1.59 (3 H, s, isopropylidene- CH_3), 1.62 (3 H, s, isopropylidene- CH_3), 3.69 (1 H, m, H-4''), 3.73 (1 H, dd, $J = 11.3$, 4.5 Hz, H-5'), 3.83–3.87 (2 H, m, H-5', H-5''), 3.90 (1 H, br, OH), 4.07 (1 H, dd, $J = 10.4$, 2.7 Hz, H-5''), 4.39 (1 H, m, H-4'), 4.87 (1 H, d, $J = 5.0$ Hz, H-2'), 4.94–4.96 (2 H, m, H-3', H-3''), 5.28 (1 H, dd, $J = 6.3$, 2.3 Hz, H-2'), 6.13 (1 H, d, $J = 2.3$ Hz, H-1'), 6.16 (1 H, br, H-1''), 7.49 (1 H, d, $J = 9.1$ Hz, NH), 7.97 (1 H, s, H-2), 8.45 (1 H, s, H-8); ^{13}C -NMR (125 MHz, CDCl_3) δ -5.64, -5.51, 18.23, 24.36, 25.27, 25.80, 26.50, 27.22, 57.20, 63.68, 64.31, 64.63, 81.06, 85.99, 86.45, 87.57, 91.20, 91.65, 95.13, 110.33, 113.74, 117.26, 132.20, 148.52, 151.04; UV (MeOH) $\lambda_{\text{max}} = 273$ nm; HR-MS (FAB, positive) calcd for $\text{C}_{27}\text{H}_{44}\text{N}_5\text{O}_7\text{SSi}$ 610.2731 (MH^+), found 610.2741.

M1-(2,3-O-isopropylidene-4-thio-5-O-dimethoxytrityl- β -D-ribofuranosyl)-5'-O-(*tert*-butyldimethylsilyl)-2',3'-O-isopropylideneadenosine (14)

A solution of **6 β** (889 mg, 1.46 mmol) and DMTrCl (989 mg, 2.92 mol) in pyridine (5 mL) was stirred at room temperature for 12 h. After addition of MeOH, the resulting mixture was partitioned between EtOAc and 1 M aqueous HCl, and the organic layer was washed with H_2O and brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (silica gel, hexane/AcOEt = 6/1, 3/1, 3/2, and 2/3) to give **14** (1.08 g, 81%, white amorphous solid): ^1H -NMR (500 MHz, CDCl_3) δ 0.05 (3 H, s, Si- CH_3), 0.06 (3 H, s, Si- CH_3), 0.88 (9H, s, *tert*-butyl), 1.30 (3 H, s, isopropylidene- CH_3), 1.38 (3 H, s, isopropylidene- CH_3), 1.59 (3 H, s, isopropylidene- CH_3), 1.62 (3 H, s, isopropylidene- CH_3), 3.40–3.46 (2 H, m, H-5'' \times 2), 3.77 (1 H, m, H-5'), 3.78 (3 H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.80–3.85 (2 H, m, H-4'', H-5'), 4.38 (1 H, ddd, $J = 5.7$, 4.0, 4.0 Hz, H-4'), 4.72 (1 H, dd, $J = 5.7$, 4.0 Hz, H-3''), 4.86 (1 H, dd, $J = 6.3$, 5.7 Hz, H-3'), 4.89 (1 H, dd, $J = 5.7$, 2.3 Hz, H-2''), 4.97 (1 H, dd, $J = 6.3$,

2.9 Hz, H-2') 6.02 (1 H, d, $J = 2.9$ Hz, H-1'), 6.52 (1 H, d, $J = 2.3$ Hz, H-1''), 6.81–7.47 (13 H, m, Ar), 7.82 (1 H, s, H-8), 8.12 (1 H, s, H-2); ^{13}C -NMR (125 MHz, CDCl_3) δ -5.58, -5.47, 18.22, 25.21, 25.28, 25.79, 27.13, 27.35, 54.95, 55.05, 63.30, 64.85, 66.78, 81.06, 84.82, 85.25, 86.53, 86.79, 89.22, 90.86, 112.38, 113.03, 113.05, 114.01, 123.23, 126.66, 127.73, 127.95, 129.92, 129.96, 135.46, 135.60, 136.51, 140.35, 144.47, 145.55, 153.98, 158.37; LR-MS (FAB, positive) m/z 882 [(M+H) $^+$]; UV (MeOH) $\lambda_{\text{max}} = 259$ nm; Anal. Calcd for $\text{C}_{48}\text{H}_{61}\text{N}_5\text{O}_9\text{SSi}$: C, 63.20; H, 6.74, N, 7.68. Found C, 63.19; H, 6.91; N, 7.39.

M1-(2,3-O-isopropylidene-4-thio-5-O-dimethoxytrityl- β -D-ribofuranosyl)-2',3'-O-isopropylideneadenosine (15)

A solution of **14** (94 mg, 103 mmol), TBAF (1.0 M in THF, 200 mL, 0.20 mmol) and AcOH (6 μL , 1 μmol) in THF (800 mL) was stirred at room temperature for 9 h, and then evaporated. The residue was purified by column chromatography (silica gel, hexane/AcOEt = 2/1, 1/1, 3/2, and 1/3) to give **15** (83 mg, quant., colorless amorphous solid): ^1H -NMR (500 MHz, CDCl_3) δ 1.29 (3 H, s, isopropylidene), 1.37 (3 H, s, isopropylidene), 1.58 (3 H, s, isopropylidene), 1.63 (3 H, s, isopropylidene), 3.40 (1 H, dd, $J = 9.7$, 6.9 Hz, H-5''), 3.46 (1 H, dd, $J = 9.7$, 6.9 Hz, H-5''), 3.72 (1 H, dd, $J = 12.6$, 10.9 Hz, H-5''), 3.77 (3 H, s, OCH_3), 3.77 (3 H, s, OCH_3), 3.79 (1 H, m, H-4''), 3.86 (1 H, d, $J = 12.6$ Hz, H-5'), 4.47 (1 H, m, H-4'), 4.67 (1 H, dd, $J = 5.7$, 4.0 Hz, H-3''), 4.84 (1 H, dd, $J = 5.7$, 2.3 Hz, H-2''), 4.99–5.03 (2 H, m, H-2', H-3'), 5.31 (1 H, d, $J = 10.9$ Hz, OH), 5.75 (1 H, d, $J = 4.6$ Hz, H-1'), 6.44 (1 H, d, $J = 2.3$ Hz, H-1''), 6.80–7.42 (13H, m, Ar), 7.57 (1 H, br s, NH), 7.63 (1 H, s, H-8), 8.14 (1 H, s, H-2); ^{13}C -NMR (125 MHz, CDCl_3) δ 25.42, 25.48, 27.60, 27.76, 54.98, 55.39, 63.28, 65.05, 67.35, 81.62, 83.90, 84.89, 86.03, 86.90, 89.28, 93.95, 112.90, 113.35, 114.39, 125.04, 127.03, 128.07, 128.26, 130.18, 130.23, 135.74, 135.92, 138.32, 139.63, 144.66, 146.01, 153.86, 158.70; UV (MeOH) $\lambda_{\text{max}} = 258$ nm; HR-MS (FAB, positive) calcd for $\text{C}_{42}\text{H}_{48}\text{N}_5\text{O}_9\text{S}$ 798.3173 [(M+H) $^+$], found 798.3193.

M1-(2,3-O-isopropylidene-4-thio-5-O-dimethoxytrityl- β -D-ribofuranosyl)-5'-O-[bis(phenylthio)phosphoryl]-2',3'-O-isopropylideneadenosine (16)

To a solution of **15** (845 mg, 1.06 mmol) in pyridine (5 mL) was added a solution of PSS (809 mg, 2.12 mmol) and TPSCl (693 mg, 1.91 mmol) in pyridine (5 mL) at -15°C , and the mixture was stirred at the same temperature for 2.5 h. After addition of H_2O , the resulting mixture was partitioned between EtOAc and 1 M aqueous HCl, and the organic layer was washed with H_2O and brine,

dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (silica gel, hexane/AcOEt = 3/1, 1/1, 1/2, and 1/3) to give **16** (812 mg, 72%, white amorphous solid): $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ 1.25 (3 H, s, isopropylidene- CH_3), 1.36 (3 H, s, isopropylidene- CH_3), 1.57 (3 H, s, isopropylidene- CH_3), 1.61 (3 H, s, isopropylidene- CH_3), 3.40 (1 H, m, H-5'), 3.46 (1 H, dd, $J = 9.2, 5.7$ Hz, H-5'), 3.77 (3 H, s, OCH_3), 3.77 (3 H, s, OCH_3), 3.79 (1 H, m, H-4''), 4.37–4.39 (2 H, m, H-5'' \times 2), 4.44 (1 H, m, H-4'), 4.64 (1 H, m, H-3''), 4.83 (1 H, dd, $J = 6.3, 2.3$ Hz, H-2''), 4.89 (1 H, dd, $J = 6.3, 3.4$ Hz, H-3'), 5.06 (1 H, dd, $J = 6.3, 2.3$ Hz, H-2'), 5.97 (1 H, d, $J = 2.3$ Hz, H-1'), 6.55 (1 H, d, $J = 2.3$ Hz, H-1''), 6.80–7.48 (23 H, m, Ar), 7.82 (1 H, s, H-8), 8.09 (1 H, s, H-2); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ 25.32, 27.14, 27.48, 55.09, 55.19, 64.99, 66.14, 66.33, 66.39, 80.89, 84.44, 84.70, 84.79, 86.58, 89.09, 90.62, 112.59, 113.12, 113.15, 123.71, 125.67, 125.71, 125.73, 125.76, 126.79, 127.85, 128.10, 129.42, 129.44, 129.63, 129.66, 129.68, 129.70, 130.03, 130.08, 135.11, 135.16, 135.27, 135.31, 135.58, 135.71, 137.27, 140.28, 144.55, 145.85, 153.96, 158.47; $^{31}\text{P-NMR}$ (202 MHz, CDCl_3) δ 50.79 (s); UV (MeOH) $\lambda_{\text{max}} = 258$ nm; HR-MS (FAB, positive) calcd for $\text{C}_{54}\text{H}_{57}\text{N}_5\text{O}_{10}\text{P}_3$ 1062.3005 $[(\text{M}+\text{H})^+]$, found 1062.2999.

M1-(2,3-O-isopropylidene-4-thio- β -D-ribofuranosyl)-5'-O-[bis(phenylthio)phosphoryl]-2',3'-O-Isopropylideneadenosine (17)

A solution of **16** (23 mg, 22 μmol) in aqueous 60% AcOH (2 mL) was stirred at room temperature for 4 h, and then evaporated. The residue was purified by column chromatography (silica gel, hexane/AcOEt = 1/1, 1/3, then AcOEt) to give **17** (15 mg, colorless amorphous solid): $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ 1.34 (3 H, s, isopropylidene- CH_3), 1.38 (3 H, s, isopropylidene- CH_3), 1.60 (3 H, s, isopropylidene- CH_3), 1.62 (3 H, s, isopropylidene- CH_3), 3.76–3.83 (3 H, m, H-4'', H-5'' \times 2), 4.30 (1 H, ddd, $J = 11.4, 9.7, 5.2$ Hz, H-5'), 4.43 (1 H, ddd, $J = 6.3, 5.2, 2.9$ Hz, H-4'), 4.47 (ddd, 1 H, $J = 12.6, 9.7, 6.3$ Hz, H-5'), 4.83 (1 H, d, $J = 5.2$ Hz, H-3''), 5.04 (1 H, dd, $J = 6.3, 2.9$ Hz, H-3'), 5.09 (1 H, dd, $J = 5.2, 1.1$ Hz, H-2'') 5.28 (1 H, dd, $J = 6.3, 1.7$ Hz, H-2'), 5.98 (1 H, d, $J = 1.7$ Hz, H-1'), 6.07 (1 H, d, $J = 1.1$ Hz, H-1''), 7.27–7.54 (10H, m, Ar), 7.63 (1 H, s, H-8), 8.55 (1 H, s, H-2); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ 24.88, 25.08, 26.87, 27.28, 57.09, 64.26, 66.26, 66.32, 73.96, 81.93, 84.52, 85.65, 85.73, 85.93, 89.68, 91.39, 110.99, 114.10, 123.84, 125.28, 125.34, 125.46, 125.52, 129.27, 129.29, 129.38, 129.40, 129.58, 129.60, 129.73, 129.75, 135.04, 135.09, 135.37, 135.40, 138.01, 140.22, 147.09, 154.09; $^{31}\text{P-NMR}$ (202 MHz, CDCl_3) δ 52.23 (s); UV (MeOH) $\lambda_{\text{max}} = 251, 258$ nm; HR-MS (FAB, positive) calcd for $\text{C}_{33}\text{H}_{39}\text{N}_5\text{O}_8\text{P}_3$ 760.1698 $[(\text{M}+\text{H})^+]$, found 760.1710.

M1-(2,3-O-isopropylidene-4-thio-5-O-phosphoryl- β -D-ribofuranosyl)-5'-O-(phenylthiophosphoryl)-2',3'-O-Isopropylideneadenosine (5)

A solution of MeOPOCl_2 (30 mL, 0.30 mmol) in pyridine (1 mL) was stirred at -15°C for 15 min. To the solution was added a solution of **17** (75 mg, 0.10 mmol) in pyridine (1 mL), and the mixture was stirred at the same temperature for 2 h. To the resulting solution was added triethylammonium acetate (TEAA) buffer (0.5 M, pH 7.0, 3 mL) then H_3PO_2 (101 μL , 2.0 mmol) and Et_3N (140 μL , 1.0 mmol), and the mixture was stirred at room temperature for 13 h, and then evaporated. The residue was partitioned between EtOAc and H_2O , and the aqueous layer was evaporated. The residue was purified by column chromatography (ODS, 1.2×16 cm, 0–37% $\text{CH}_3\text{CN}/0.1$ M TEAA buffer (0.1 M, pH 7.0, 400 mL), linear gradient). The excess TEAA included in the residue was removed by column chromatography (ODS, 1.2×16 cm, $\text{CH}_3\text{CN}/\text{H}_2\text{O} = 1/1$). The product was lyophilized to give **5** (37 mg, 512 OD_{260} unit, 46%) as a triethylammonium salt: $^1\text{H-NMR}$ (500 MHz, D_2O) δ 1.26 (9H, t, $J = 7.4$ Hz (CH_3CH_2) $_3\text{N}$), 1.40 (3 H, s, isopropylidene- CH_3), 1.43 (3H, s, isopropylidene- CH_3), 1.63 (3 H, s, isopropylidene- CH_3), 1.69 (3H, s, isopropylidene- CH_3), 3.18 (6H, q, $J = 7.4$ Hz, (CH_3CH_2) $_3\text{N}$), 4.10–4.13 (4H, m, H-4'', H-5' \times 2, H-5''), 4.70 (1 H, m, H-4''), 4.20 (1 H, m, H-5'), 4.22 (1 H, m, H-5''), 4.32 (1 H, m, H-5''), 4.71 (1 H, m, H-4'), 4.94 (1 H, d, $J = 5.2$ Hz, H-3') 5.12–5.15 (2 H, m, H-2'', H-3''), 5.42 (1 H, dd, $J = 6.3, 2.9$ Hz, H-3'), 5.39 (1 H, d, $J = 5.2$ Hz, H-2'), 5.95 (1 H, s, H-1''), 6.37 (1 H, s, H-1'), 7.19–7.33 (5H, m, Ar), 8.41 (1 H, s, 8-H), 9.24 (1 H, s, 2-H); $^{13}\text{C-NMR}$ (125 MHz, D_2O) δ 8.30, 24.32, 24.35, 25.95, 26.41, 46.71, 54.48, 54.55, 65.90, 65.95, 66.59, 66.62, 75.84, 81.39, 84.11, 86.19, 86.27, 86.52, 89.30, 90.88, 113.51, 114.72, 119.08, 127.91, 129.08, 129.47, 129.51, 132.75, 132.79, 143.02, 145.48, 146.84, 150.53; $^{31}\text{P-NMR}$ (202 MHz, D_2O) δ 17.54 (s), 0.80 (s); UV (D_2O) $\lambda_{\text{max}} = 258$ nm; HR-MS (FAB, negative) calcd for $\text{C}_{27}\text{H}_{34}\text{N}_5\text{O}_{12}\text{P}_2\text{S}_2$ 746.1126 $[(\text{M}-\text{H})^-]$, found 746.1106.

Cyclic ADP-4-Thio-Ribose 2',3'-, 2'',3''-Bisacetone (19)

To a mixture of AgNO_3 (36 mg, 0.21 mmol), Et_3N (29 mL, 0.21 mmol), and MS 3A (powder, 1.0 g) in pyridine (8 mL), a solution of **5** (9 mg, 50 OD_{260} unit, 4 mmol) in pyridine (8 mL) was added slowly over 15 h, using a syringe-pump, at room temperature under shading. To the mixture was added TEAA buffer (2.0 M, pH 7.0, 2 mL), and the resulting mixture was filtered with Celite and evaporated. The residue was partitioned between EtOAc and H_2O , and the aqueous layer was evaporated, and the residue was purified by column chromatography (ODS, 1.2×16 cm, 0–35% $\text{CH}_3\text{CN}/0.1$ M

TEAA buffer (0.1 M, pH 7.0, 400 mL), linear gradient). The excess TEAA included in the residue was removed by column chromatography (ODS, 1.2×16 cm, $\text{CH}_3\text{CN}/\text{H}_2\text{O} = 1/1$). The product was lyophilized to give **19** (6 mg, 36 OD₂₆₀ unit, 72%) as a triethylammonium salt: ¹H-NMR (500 MHz, D₂O) δ 1.24 (9H, t, $J = 7.4$ Hz (CH_3CH_2)₃N), 1.41 (3 H, s, isopropylidene-CH₃), 1.43 (3 H, s, isopropylidene-CH₃), 1.61 (3 H, s, isopropylidene-CH₃), 1.65 (3 H, s, isopropylidene-CH₃), 3.17 (6H, q, $J = 7.4$ Hz, (CH_3CH_2)₃N), 3.91 (1 H, m, H-5'), 4.13 (1 H, m, H-4''), 4.20 (1 H, m, H-5'), 4.22 (1 H, m, H-5''), 4.32 (1 H, m, H-5''), 4.58 (1 H, m, H-4'), 5.07 (1 H, d, $J = 4.8$ Hz, H-3''), 5.13 (1 H, d, $J = 4.8$ Hz, H-2''), 5.42 (1 H, dd, $J = 6.3, 2.9$ Hz, H-3'), 5.89 (1 H, dd, $J = 6.3, 1.7$ Hz, H-2'), 5.99 (1 H, s, H-1''), 6.38 (1 H, d, $J = 1.7$ Hz, H-1'), 8.38 (1 H, s, 8-H), 9.59 (1 H, s, 2-H); ¹³C-NMR (125 MHz, D₂O) δ 8.29, 24.39, 24.42, 26.07, 26.26, 46.73, 55.40, 55.49, 64.80, 68.03, 64.83, 68.00, 77.65, 81.36, 83.25, 86.65, 86.73, 87.34, 90.82, 91.45, 113.11, 114.59, 119.58, 144.91, 145.55, 147.20, 150.67; ³¹P-NMR (202 MHz, D₂O) δ -10.62 (d, $J = 15.5$ Hz), δ -11.29 (d, $J = 15.5$ Hz); UV (D₂O) $\lambda_{\text{max}} = 258$ nm; HR-MS (FAB, negative) calcd for C₂₁H₂₈N₅O₁₂P₂S 636.0936 [(M-H)⁻], found 636.0947.

Cyclic ADP-4-Thio-Ribose (3)

A solution of **19** (280 OD₂₆₀ unit, 25 mmol) in aqueous 60% HCO₂H (1 mL) was stirred at room temperature for 40 h and then evaporated. After co-evaporation with H₂O, the residue was purified by column chromatography (ODS, 1.2×16 cm, TEAA buffer (5 mM, pH 7.0)). The excess TEAA included in the residue was removed by column chromatography (Sephadex LH 20, 2.5×20 cm, H₂O) to give **3** as a triethylammonium salt, which was converted into a free acid form by passing through columns of Diaion PK212L (H⁺ form, 0.7 cm \times 5 cm, H₂O). The free acid was passed through Chelex 100 column (K⁺ form, 0.7 cm \times 6 cm, H₂O). The eluent was evaporated and lyophilized to give **3** (138 OD₂₆₀ unit, 49%) as a potassium salt. **3** (potassium salt): ¹H-NMR (500 MHz, D₂O) δ 3.59 (1 H, dd, $J = 8.2, 3.2$ Hz), 4.08 (1 H, m), 4.19 (1 H, m), 4.24–4.36 (4H, m), 4.52 (1 H, ddd, $J = 10.4, 8.2, 2.7$ Hz), 4.60 (1 H, dd, $J = 5.4, 2.3$ Hz), 5.18 (1 H, dd, $J = 5.9, 5.4$ Hz), 5.87 (2 H, m), 7.97 (1 H, s), 9.25 (1 H, s); ¹³C-NMR (125 MHz, D₂O) δ 50.8, 50.9, 63.1, 65.0, 65.0, 70.1, 70.6, 72.6, 73.0, 78.3, 84.7, 84.8, 90.5, 120.0, 145.3, 145.9, 146.9, 150.8; ³¹P-NMR (162 MHz, D₂O) δ -9.15 (d, $J = 12.2$ Hz), δ -10.20 (d, $J = 12.2$ Hz); UV (D₂O) $\lambda_{\text{max}} = 258$ nm; HR-MS (FAB, negative) calcd for C₁₅H₂₀N₅O₁₂P₂S 556.0304 [(M-H)⁻], found 556.2994. **3** (free acid): ¹H-NMR (500 MHz, D₂O) δ 3.72 (1 H, ddd, $J = 5.2, 3.4, 1.7$ Hz), 4.09 (1 H, ddd, $J = 10.9, 6.9, 2.9$ Hz), 4.25 (1 H, ddd, $J = 11.5, 5.2, 2.3$ Hz), 4.35–4.39 (3 H, m), 4.51 (1 H, ddd, $J = 10.9, 7.4, 4.0$ Hz), 4.53

(1 H, dd, $J = 5.9, 2.9$ Hz), 4.71 (1 H, dd, $J = 5.2, 2.3$ Hz), 5.22 (1 H, dd, $J = 6.3, 5.2$ Hz), 5.97 (1 H, d, $J = 2.9$ Hz), 6.04 (1 H, d, $J = 6.3$ Hz), 8.38 (1 H, s), 9.90 (1 H, s).

Stability in Rat Brain Microsomes

Rat brain microsomes were prepared by a procedure according to the previous method (Murayama, T. and Ogawa Y. (1996). *J. Biol. Chem.* 271, 5079–5084.). cADPR or cADPr (1.6 OD₂₆₀ unit) was preincubated in 20 mM MOPS buffer (pH 7.1, 160 μ L) at 37 °C for 5 min. This was added to the solution of the microsome fraction of rat brain extract (14.9 mg/mL, 140 μ L), and the mixture was incubated at 37 °C. The reaction mixture was sampled (25 μ L) at every 30 min afterwards and diluted with water (175 μ L), which was frozen in liquid nitrogen to stop the reaction. After the samples were a centrifuged at 12000 rpm at 4 °C for 15 min, the supernatants were filtered using centrifugal filter at 12000 rpm at 4 °C for 15 min, and the resulting filtrates (70 μ L) were analyzed by ion exchange HPLC (TSK-GEL DEAE-2SW, 4.6×250 mm; 5–35% 1 M HCO₂NH₄/20% MeCN, 20 min; 260 nm). The results are shown in Figure 2.

Biological Evaluations with Sea Urchin Egg Homogenate or T-cells

These bioassays were carried out as reported previously (Kudoh et al., 2005).

Biological Evaluations with Neuronal Cultured Cells

NG108-15 neuroblastoma \times glioma hybrid cells were cultured as reported previously (Higashida et al., 1990). Oregon Green-loaded NG108-15 cells were incubated for 2 min in the following calcium-free medium (140 mM K-glutamate, 20 mM PIPES, 5 mM EGTA, 2 mM Mg-ATP, 10 mM glucose, 1 mM magnesium chloride, 0.01% bovine serum albumin, pH 6.8) at 37 °C, and subsequently permeabilized with 250 nM digitonin in the calcium-free medium. cADPR (1–100 μ M), cADPr (1–100 μ M), or cADPr (1–100 μ M) were applied together with the digitonin-containing permeabilization buffer to be allowed free passage of these nucleotides into the cytoplasm. Concentrations of $[\text{Ca}^{2+}]_i$ were determined microspectrofluorometrically using fura-2 in differentiated NG108-15 cells cultured on polylysine-coated glass coverslips. The cells were loaded with fura-2 using 5 μ M Oregon Green 488 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetate acetoxymethylester (BAPTA-1 AM). Fluorescence was measured at 37 °C with excitation wavelengths of 485 nm and emission wavelengths of 538 nm using an Argus 50 Ca²⁺ microspectrofluorometric system (Hamamatsu Photonics, Hamamatsu, Japan) and images were collected every 10 s for up to 5 min.

The changes in fluorescence intensity of each cell were expanded into an X-t plane, and data were performed in fluorescence intensity at each time (x) divided by resting intensity at time 0, i.e., F_x/F_0 (Amina et al., 2010). Data are expressed as mean \pm s.e.m. Statistical analysis was performed using a Student's t test. The criterion for significance in all cases was $p < 0.05$.

Computational Calculations

For structure determination, molecular calculations were carried out by AMBER11 (Case et al., 2010). cADPR (1), cADPcR (2) and cADPtR (3) were modeled by the General AMBER Force Field (Wang, et al., 2004). AM1-BCC charge (Jakalian, A., Jack, D. B., and Bayly, C. I. (2002). *J. Comput. Chem.* 23, 1623–1641) was used for cADPR and its analogs assigned by Antechamber module (Wang et al., 2006) of AMBER11. In the calculations, interatomic distances restricts were determined by the integrated volumes of the NOESY cross-peaks. As cross peaks of cADPR and cADPcR, previously published data were used (Kudoh, et al., 2005). For generating conformations, we carried out 400 ps simulated annealing molecular dynamics simulations: temperature was decreased from 1000 to 300 K. These simulations were repeated 100 times with different initial velocity for each compound. In calculated conformations, we chose and analyzed the lowest energy conformation.

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